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☐ *Additional inventors are being named on the ___ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

METHODS FOR INDUCING AN IMMUNE RESPONSE VIA ORAL ADMINISTRATION OF AN ADENOVIRUS

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☒ Specification *Number of Pages*	30	☐ CD(s), Number	
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Respectfully submitted,

SIGNATURE Jane Massey Licata Date 6/18/03

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Jane Massey Licata
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METHODS FOR INDUCING AN IMMUNE RESPONSE VIA ORAL ADMINISTRATION OF AN ADENOVIRUS

Introduction

This invention was made in the course of research sponsored by the National Institutes of Health (NIAID Grant No. P01A1052271). The U.S. government may have certain rights in this invention.

Background of the Invention

Vaccines remain an efficacious medical intervention to reduce mortality and morbidity due to pathogens. While more than 400 distinct viruses can cause symptomatic infections in humans, prophylactic vaccines are available for less than 20 of these pathogens.

Traditionally, vaccines have been developed by inactivation or attenuation of pathogens. Advances in molecular biology now allow for the generation of recombinant subunit vaccines based on different carriers, which impact the magnitude and the type of the immune response to the vaccine antigen. The type of the vaccine vehicle also imposes constraints on the potential routes of vaccine delivery.

Adenoviral (Ad) recombinants of the human serotype 5 (Hu5) are efficacious as vaccine carriers in experimental animals (He, et al. (2000) *Virology* 270:146-161; Moraes, et al. (2002) *Vaccine* 20:1631-1639; Shiver, et al. (2002) *Nature* 415:331-335; Sullivan, et al. (2000) *Nature* 408:605-609; Tims, et al. (2001) *Vaccine* 18:2804-2807; Xiang, et al. (1996) *Virology* 219:220-227) and are now in clinical trials (Mincheff, et al. (2001) *Crit. Rev. Oncol. Hematol.* 39:125-132) and have been administered by various routes (Vos, et al. (2001) *J. Gen. Virol.* 82:2191-2197).

Intranasal application of such vaccines has been tested (Gogev, et al. (2002) *Vaccine* 20:1451-1465; Xiang and Ertl (1999) *Vaccine* 17: 2003-2008) and shown to induce antibody responses at mucosal surfaces, the most common port of entry for most viral pathogens. Replication-defective or replication-competent Ad recombinants of human, or porcine serotypes have been demonstrated to induce cellular and humoral immunity to the target antigen upon oral or enteric administration (Hammond, et al. (2001) *Arch. Virol.* 146:1787-1793; Mutwiri, et al. (2000) *Vaccine* 19:1284-1293; Sharpe, et al. (2002) *Virology* 293:210-216; Vos et al. (2001) *J. Gen. Virol.* 82:2191-7). Further, oral administration of an AdHu5 vaccine expressing rabies glycoprotein G overcomes immunity against canine adenovirus in fox (Vos, et al. (2002) *supra*). Epicutenous application through dermal patches have been used, however with limited success (Lees, et al. (2002) *Vet. Microbiol.* 85:295-303; Shi, et al. (2001) *J. Virol.* 75:11474-11482).

Vaccine carriers that achieve protective immune responses upon oral immunization are needed for several reasons. Vaccines that can be given through the oral route are highly desirable for developing countries where a lack of skilled medical personnel and insufficient resources cause logistic problems for mass vaccinations given by injection. Repeated use of unsterile needles can lead to inadvertent spread of other human pathogens such as HIV-1, thus negating the benefit of vaccination (Jodar, et al. (2001) *Vaccine* 19:1594-1605). In developed countries facing an increased risk of intentional release of pathogens (Gostin, et al. (2002) *J. Am. Med. Assoc.* 288:622-628), oral vaccines would allow for a more rapid mass vaccination than could be achieved by vaccines applied by injection or by propulsion devices. In addition, mucosal vaccination

such as intranasal or oral vaccination favors the induction of antibodies secreted at mucosal surfaces (Xiang and Ertl (1999) *supra*), which are common ports of entry for the invasion of many pathogens including those that spread through aerosoles or by sexual contact. While intranasal vaccination is cumbersome and difficult to dose, oral vaccination has proven highly successful in the poliovirus eradication campaign (Sabin (1965) *J. Am. Med. Assoc.* 194:872-876). Further, U.S. Patent No. 6,348,450 discloses inducing an immune response to an adenovirus by topically administrating an adenoviral vector containing a gene of interest to the skin of a mammal. While this patent indicates that an adenovirus may be administered orally, the teachings primarily focus on administration of an adenovirus to external skin surfaces and the oral and nasal cavities.

Vaccines to viral pathogens, which can be distributed rapidly to large segments of a susceptible population, are needed. The present invention meets this long-felt need.

Summary of the Invention

One aspect of the present invention is a method for inducing an immune response in a subject pre-exposed to an adenovirus. The method involves orally administering to a subject, that has been exposed to a first adenovirus, an effective amount of a second adenovirus so that an immune response to the second adenovirus is induced. In a preferred embodiment, the first and second adenovirus contain a nucleic acid sequence encoding an antigen to which the immune response is directed. In another preferred embodiment, the first adenovirus and the second adenovirus are part of a vaccine.

Another aspect of the present invention is a method for inducing a mucosal immune response to an antigen. The method involves orally administering an effective amount of a first adenovirus containing nucleic acid sequences encoding an antigen, and orally administering an effective amount of a second adenovirus containing said nucleic acid sequences encoding said antigen, so that a mucosal immune response to said antigen is induced. In a preferred embodiment of the present invention, the first adenovirus and the second adenovirus are homologous. In another preferred embodiment, the first adenovirus and the second adenovirus are heterologous. In a further preferred embodiment, the first adenovirus and second adenovirus comprise a vaccine.

Detailed Description of the Invention

Rabies virus, a simple RNA virus, is well-defined and neutralizing antibodies against the viral glycoprotein, are known (Sullivan, et al. (2000) *supra*). Animal models, including those based on rodents, are considered valid for pre-clinical vaccine testing and have been used to evaluate novel vaccine carriers or adjuvants that aim to induce neutralizing antibody responses to the vaccine antigen (Xiang and Ertl (1999) *supra;* Xiang, et al. (2002) *J. Virol.* 76:2667-2675; Xiang, et al. (1994) *Virology* 199:132-140; Xiang, et al. (1996) *supra*). To reflect the genetic diversity of the human population, the studies provided herein used outbred ICR mice in addition to better-characterized inbred strains of mice.

AdHu5 virus, the most commonly used vector for pre-clinical vaccine studies is a ubiquitous pathogen, and circulating serotype-specific neutralizing antibodies found in up to 45% of the adult United States population

interfere with the efficacy of systemically delivered Ad vaccines based on the homologous serotype (Farina, et al. (2001) *J. Virol.* 75:11603-11613; Moffatt, et al. (2000) *Virology* 272:159-167; Papp, et al. (1999) *Vaccine* 17:933-943; Xiang, et al. (2002) *supra*). An alternative vector system based on an Ad virus originating from the lymph nodes of a chimpanzee was developed. E1-deleted recombinants derived from this virus, designated chimpanzee serotype 68 (AdC68), induce in rodents upon systemic or intranasal application a transgene product-specific antibody response, which is not impacted by pre-existing immunity to common human serotypes of Ad virus (Xiang, et al. (2002) *supra*). These studies were conducted in a mouse rabies virus model considered an appropriate model for human rabies vaccines. Current vaccine lots are analyzed by a potency test in rodents (Fitzgerald, et al. (1978) *Dev. Biol. Stand.* 40:183-186), before release for use in humans. Rabies virus is a simple RNA virus encoding five antigens. Of those, the glycoprotein is the sole target of virus neutralizing antibodies (VNAs), which provide protection to viral challenge (Xiang, et al. (1995) *Virology* 214:398-404).

It has now been found that oral delivery (os) of E1-deleted Ad vectors of the serotypes Hu5 and C68 expressing the glycoprotein of the fixed Evelyn Rokitniki Abelseth (ERA) strain of rabies virus stimulate a systemic and mucosal antibody response and protection to a severe rabies virus challenge. In addition, it was found that transgene product-specific humoral immune response to oral Ad vaccination was not strongly impaired by pre-existing antibodies to the vaccine carrier and this response could be boosted by a second dose of the homologous vaccine carrier again given per os.

E1-deleted Ad vectors of human and simian serotypes induce transgene product-specific serum antibodies upon oral application. The rabies virus glycoprotein (rab.gp) was used to test for the induction of antibodies by Ad recombinants based on the human serotype 5 (AdHu5rab.gp) or the chimpanzee serotype 68 (AdC68rab.gp). It is known that that upon subcutaneous (s.c.) or intramuscular (i.m.) immunization both vaccines stimulate antibodies to rabies virus, although serum titers are markedly higher upon vaccination with the AdHu5rab.gp vector. Upon intranasal (i.n) immunization, both vaccines induce more comparable titers of rabies virus-specific antibodies in sera (Xiang and Ertl (1999) *supra*). Thus, oral immunization of outbred ICR and inbred C57Bl/6 mice with escalating doses of the AdHu5rab.gp and the AdC68rab.gp vectors was evaluated. In general, the latter strain of mice mount a less vigorous B-cell response to the rabies virus glycoprotein compared to other mouse strains such as ICR or C3H/He mice. The results of these studied showed mice of either strain developed antibody titers to rabies virus at doses of or above 2 x 10^6 pfu. Oral immunization was not as effective as i.m. vaccination for the AdHu5rab.gp vector applied at high (10^7 pfu) or low (10^5 pfu) doses to groups of ICR mice.

Sera from ICR mice orally vaccinated with AdHu5rab.gp or AdC68rab.gp were tested for rabies virus-specific VNAs which are important for protection against virus infection (Xiang, et al. (1995) *supra*). Both vaccines induced serum VNA responses to rabies virus upon oral application and correspondingly protective immunity to rabies virus challenge given directly into the central nervous system. VNA titers and protective immunity, unlike titers tested by ELISA, showed a dose response curve for both vaccines. Upon systemic immunization with recombinant vaccines, titers

detected by ELISA correlate with those determined by neutralization assays (Xiang, et al. (2002) *supra;* Xiang, et al. (1996) *supra;* Xiang, et al. (1994) *Virology* 199:132-140). These results indicate that antibodies elicited against the vector-encoded viral protein were directed against epitopes expressed on correctly folded protein and that these antibodies possess neutralizing activity. Upon oral immunization, this correlation was less rigorous indicating that the B-cell response may have targeted, in part, unfolded or partially degraded rabies virus glycoprotein. Thus, resulting in a high fraction of non-neutralizing antibodies that detected by the ELISA. While both vaccines had reduced efficacy via oral immunization compared to systemic routes of immunization, complete protection could be achieved with either vaccine upon oral application of 2×10^7 pfu of the vectors.

It has been shown that, upon s.c. immunization, the Adhu5rab.gp virus induced a mixed Th1/Th2 response with a ratio of IgG2a/IgG1 of approximately two, while the AdC68rab.gp virus favored stimulation of a Th1 response providing a ratio of IgG2a/IgG1 of approximately 10 (Xiang, et al. (2002) *supra*). This difference in isotype distribution of the transgene product-specific antibodies is not observed upon intranasal immunization (Sharpe, et al. (2002) *Virology* 293:210-216) or upon oral vaccine application, as the results herein indicate.

Upon intramuscular injection of the Ad recombinants, lymph nodes draining the injection sites rapidly, within less than 24 hours, acquire transgene product expressing cells with morphological and phenotypic characteristics of mature dendritic cells. These cells may become infected at an immature stage at the site of inoculation and then upon maturation migrate to lymphatic tissues where they present

the antigen to naïve T-cells. To determine which lymphatic tissues became infiltrated by recombinant Ad virus infected migratory cells and thus were likely to participate in induction of an immune response upon oral administration of the Ad vectors, mice were fed 1 x 10^8 pfu of AdHu5rab.gp or AdC68rab.gp virus. Lymph nodes (cervical and mesenteric) and Peyers' Patches were harvested at 18, 48 or 72 hours after administration, RNA was isolated and subsequently reverse transcribed and PCR-amplified for rabies virus glycoprotein- and GAPDH-specific cDNA. Rabies virus-specific amplicons were detected in all of the lymph nodes at least at one of the time points indicating that the vaccines had been taken up within the oral cavity as well as within the intestine.

It was found that E1-deleted adenoviral vaccines induce mucosal antibody responses upon oral application. Mucosal immunization such as through the oral or respiratory routes favors induction of antibodies secreted at mucosal surfaces. This was further analyzed by feeding ICR mice either the AdHu5rab.gp or the AdC68rab.gp vaccine. With oral vaccination of either vaccine, antibodies titers to rabies at vaginal mucosa and in fecal suspensions were comparable in outbred ICR mice.

Oral vaccination overcame interference by pre-existing neutralizing antibodies to the Ad vaccine carrier. Serum antibody response to the rabies virus glycoprotein when presented by a systemic AdHu5rab.gp vaccine is strongly reduced while the antibody response to a systemic AdC68rab.gp vaccine is not impaired in mice pre-exposed to AdHu5 virus (Sharpe, et al. (2002) *supra*). Thus, it was determined whether the humoral response elicited by oral vaccination was able to overcome pre-existing immunity to the vaccine carrier. Mice were immunized with replication

competent (in their natural host) AdHu5 virus given at 10^9 pfu i.m. or at a lower dose of 10^8 pfu intranasally, the natural route of infection of humans by this virus. Serum antibody titers to the AdHu5 vector, tested four weeks later by a neutralization assay were ~1:160 in the i.m. vaccinated group, which is comparable to titers commonly found in human adults. Intranasally vaccinated mice had neutralizing antibody titers below 1:20 although antibodies to Ad virus could readily be detected by ELISA. AdHu5-immune, as well as naïve mice, were subsequently vaccinated with the AdHu5rab.gp vector given either per os or i.m.. Mice were bled two weeks later and serum antibody titers to rabies virus were determined by a neutralization assay. The antibody response to i.m. vaccination with the AdHu5rab.gp vector, which induced a potent response in naïve ICR mice, was completely abrogated in mice pre-exposed by i.m. inoculation with AdHu5 virus and moderately decreased upon intranasal pre-exposure. Antibody titers to rabies virus generated upon oral immunization were overall lower in naïve mice compared to those achieved by i.m. immunization. In mice pre-immunized systemically with AdHu5 virus prior to per os vaccination with the Adhu5rab.gp vector, VNA titers to rabies virus were identical to those elicited in mice that had not been pre-exposed to the vaccine. Pre-exposure through the airways caused a slight increase in VNA titers elicited by oral vaccination. Overall these data indicate that oral immunization is relatively unaffected by pre-existing neutralizing antibodies to the vaccine carrier. Part of the experiment was repeated using different doses of the AdHu5rab.gp vector to determine whether low vaccine doses could be inhibited by pre-existing antibodies to the vaccine carrier. Intranasal pre-exposure was chosen for these experiments as this regimen

is better suited to induce mucosal antibodies of the IgA isotype (Xiang, et al. (1999) *supra*). Intranasally, pre-exposed and naïve mice were vaccinated orally with the AdHu5rab.gp vector. Two groups of mice were vaccinated with an intermediate dose of the AdC68rab.gp vector. Pre-exposure to AdHu5 virus had no effect on the transgene product-specific antibody titers in sera or at vaginal surfaces induced by the two higher doses (2×10^6 and 2×10^7 pfu per mouse) of the AdHu5rab.gp vector or the intermediate dose (2×10^6 pfu) of the AdC68rab.gp vaccine. The serum and vaginal antibody response to the lowest dose (2×10^5 pfu per mouse) of AdHu5rab.gp vaccine was marginally reduced in AdHu5 pre-exposed mice, indicating that low does oral immunization may be affected by mucosal pre-exposure to AdHu5 virus. The vaginal response to the highest dose of the AdHu5rab.gp vector, as well as to the intermediate dose of the AdC68rab.gp vector, were slightly increased in pre-immune mice indicating a potential benefit from the AdHu5 pre-exposure on the transgene product-specific mucosal B-cell response to the vaccine antigen. The VNA response in these studies, similar to the dose titration experiments, showed no strict correlation with the serum antibody titers determined by ELISA. Again, VNA titers were not strongly reduced in AdHu5 pre-exposed mice vaccinated with the high or intermediate doses of AdHu5rab.gp or AdC68rab.gp virus; the response to the intermediate dose of AdHu5rab.gp virus was slightly increased by approximately 2-fold which was within the range of assay variability. The isotype profiles of serum and vaginal antibodies to rabies virus were not affected in AdHu5rab.gp vector-fed mice pre-exposed to airway-administered AdHu5 virus. The isotype profile of vaginal antibodies to rabies virus elicited by oral administration

of the AdC68rab.gp vaccine was shifted towards IgA in AdHu5 pre-immune mice, indicative of an AdHu5-specific T-helper cell effect on the vaccine-induced B-cell response.

Oral booster immunization enhances the antibody response to the transgene product of Ad vectors. A second oral and intermediate dose (2×10^6 pfu) of either AdC68rab.gp or AdHu5rab.gp virus was administered to groups of ICR mice. Mice were boosted 4 weeks later orally with the same dose of homologous or heterologous carrier used for priming. Serum antibody responses to rabies virus glycoprotein were analyzed 2 and 8 weeks later. The oral booster immunization enhanced serum antibody responses at both time points. The AdHu5rab.gp-primed group responded with a similar increase in rabies virus-specific antibody titers to booster immunization with homologous or heterologous vaccine carrier as shown by ELISA. VNA titers indicated an advantage for the homologous booster immunization. In AdC68rab.gp-primed mice, booster immunization with the heterologous AdHu5rab.gp vector resulted in slightly higher rabies virus-specific antibody titers by ELISA and neutralization assay compared to those achieved with a second dose of the AdC68rab.gp vector. In either combination, priming with the AdHu5rab.gp vector elicited higher titers of antibodies than priming with the AdC68rab.gp vector. These results were compared with systemic prime boost regimens in which mice were immunized with a low dose (10^5 pfu) of either AdHu5rab.gp or AdC68rab.gp vector. Mice were boosted two months later with the same dose of either homologous or heterologous vaccine carrier. Control groups did not receive the second dose of vaccine. Serum antibody titers to rabies virus analyzed two weeks after the booster immunization showed high VNA titers of 1:100 IU upon a single immunization with the AdHu5rab.gp

vector. A second immunization with either the AdHu5rab.gp or the AdC68rab.gp vector failed to increase these titers. The AdC68rab.gp vector on the other hand induced at these low doses only modest VNA titers, which failed to increase upon booster immunization with the homologous construct, indicating that neutralizing antibodies to the vaccine carrier impaired uptake of the second vaccine dose. Booster immunization of AdC68rab.gp-immune mice with the AdHu5rab.gp vector on the other hand increased VNA titers to rabies virus dramatically. These data demonstrate the high susceptibility of systemic Ad vector immunization to interference by neutralizing antibodies to the vaccine carrier. The same groups of mice which were orally vaccinated in these studies were analyzed for isotypes of rabies virus specific antibodies in vaginal lavage. An unexpected difference in the effect of homologous versus heterologous prime-boost vaccination became apparent. Upon priming, vaginal lavage from mice fed the AdHu5rab.gp or the AdC68rab.gp vector contained antibodies to rabies virus that, by 2 weeks after vaccination, were mainly of the IgA isotype. Upon booster immunization of AdHu5rab.gp-primed mice with either the AdHu5rab.gp or the AdC68rab.gp vectors, mice developed within 2 weeks a pronounced IgG2a response that exceeded the IgA response. Two months after booster immunization, the rabies virus-specific antibodies in vaginal lavage of mice vaccinated twice per os with AdHu5rab.gp virus reversed to a preponderance of the IgA isotype. In contrast, in AdHu5rab.gp-primed mice boosted with the AdC68rab.gp vector, vaginal antibodies to rabies virus remained dominated by antibodies of the IgG2a isotype although levels of IgA antibodies were also substantial. AdC68rab.gp-primed mice showed low levels of IgA in their vaginal lavage 2 weeks after booster immunization with

either the homologous or the heterologous vaccine carrier and levels of IgG antibodies were marginal. After two months, mice developed a pronounced IgG2a response that exceeded the IgA response. This was particularly noticeable in mice vaccinated twice with the AdC68rab.gp vector. To further assess the apparent preference of the two viral vectors to differentially induce mucosal IgA versus IgG2a antibodies to the transgene product, mice were vaccinated in follow-up experiments with an increased dose of either vaccine given at 2×10^7 pfu per os. One month later, mice were boosted with the same dose of the homologous vectors and vaginal titers and isotypes of antibodies to rabies were determined two months later. AdHu5rab.gp-vaccinated mice developed high levels of IgA to rabies virus while this isotype was virtually absent in vaginal lavage from mice immunized twice with the AdC68rab.gp vector. Mice immunized twice with AdC68rab.gp contained predominantly rabies virus-specific antibodies of the IgG2a isotype. These results indicated oral homologous versus heterologous booster immunization with two Ad vaccine carriers had a distinct effect on the isotypes of vaginal antibodies as was observed upon pre-exposure to AdHu5 virus. Heterologous primer-boost vaccination regardless of the sequence of the vaccine carriers resulted in a balanced ratio or IgA or IgG2a antibodies (IgA:IgG2a ~0.8). Conversely, a double immunization with AdHu5rab.gp vector favored induction of vaginal IgA over IgG2a antibodies to rabies virus (IgA:IgG2a>2) while repeated oral application of the AdC68rab.gp vector strongly favored induction of vaginal IgG2a over IgA responses (IgA:IgG2a >0.3).

The unexpected efficacy of homologous oral prime boosting with the AdHu5 vector was compatible with the observed lack of interference of pre-exposure to wild-type

AdHu5 virus on the transgene product-specific antibody response to oral AdHu5rab.gp vaccination. These results indicate that, upon oral immunization, intestinal production of antibodies to the vaccine carrier is either low or short-lived and that pre-exposure or priming through intranasal or oral routes fails to affect uptake of the same vaccine carrier given several weeks later per os. It has been shown that intranasal immunization with the AdHu5 vector resulted in sustained antibody titers to the antigens of the vaccine carrier in fecal suspension (Xiang and Ertl (1999) *supra*). In similar studies conducted herein, mice vaccinated orally with 2×10^7 or 2×10^6 pfu of the AdHu5rab.gp vaccine had readily detectable antibody titers, of predominantly IgA, to the antigens of AdHu5 virus in fecal suspensions one month after the vaccination.

Accordingly, one aspect of the present invention is a method for inducing an immune response to an adenovirus in a subject pre-exposed to a first adenovirus via oral administration of a second adenovirus. By administering the second adenovirus orally, an immune response to said second adenovirus is induced. As used herein, an immune response is defined as a mucosal or systemic immune response characterized by induction of a measurable B cell response or elicitation of a T cell response (*e.g.*, T helper or cytotoxic T cells) which is brought about by exposure to an antigen (*e.g.*, adenoviral proteins or exogenous antigens expressed from an adenovirus).

An adenovirus, as used within the scope of the present invention, includes wild-type adenovirus and adenoviral vectors, *e.g.*, an adenoviral vaccine. Hence, pre-exposure to a first adenovirus is intended to include natural exposure to an adenovirus as well as exposure resulting from vaccination using an adenoviral vector. As

demonstrated herein, exposure to a first adenovirus by any route may induce an immune response to said first adenovirus. Interference by this first immune response to a second adenovirus may be overcome upon oral administration of the second adenovirus, which is preferably an adenoviral vector. The effectiveness of oral administration to mount a second immune response is such that the first and second adenovirus may or may not be of the same serotype or from the same animal origin.

When the first or second adenovirus is an adenoviral vector, it is preferred that the adenoviral vector is replication-defective, *i.e.*, a vector that is unable to replicate autonomously in a host cell. Typically, the genome of a replication-defective adenovirus lacks at least the sequences which are necessary for replication of said adenovirus in a host cell. These regions may be eliminated in whole or in part, be rendered non-functional, or be substituted by other sequences, in particular by nucleic acid sequence encoding an antigen of interest. Preferably, a replication-defective adenovirus retains the sequences of its genome which are necessary for encapsidating the viral particles.

Adenoviruses exist as various serotypes whose structure and properties differ. Of these serotypes, preference is given to using subgroup C type 2 or type 5 human adenoviruses (Ad 2 or Ad 5), chimpanzee serotype Ad C68, Ad C6, Ad C7, or other adenoviruses of animal origin. Adenoviruses of animal origin which may be used are adenoviruses of canine, bovine, murine, ovine, porcine, avian, caprine, guinea pig, fowl, fish, possum, deer or simian origin. Preferably, the adenovirus of animal origin is a simian or canine adenovirus. Preferably, use is made of adenoviruses of human, simian, canine or mixed origin.

Particularly suitable adenoviruses of animal origin are well-known to those of skill in the art.

Preferably, a replication-defective adenovirus of the invention contains inverted terminal repeats, an encapsidation sequence and a nucleic acid sequence of interest. Still more preferably, in the genome of an adenovirus of the invention, at least the E1 region is non-functional. A viral gene under consideration may be rendered non-functional by any technique known to the person skilled in the art, in particular by total removal, substitution, partial deletion or the addition of one or more bases to the genes under consideration. Such modifications may be achieved *in vitro* on isolated DNA or *in situ*, for example using techniques of genetic manipulation or by treatment with mutagenic agents. Other regions may also be modified, in particular the E3 region (WO95/02697), the E2 region (WO94/2938), the E4 region (WO94/28152, WO94/12649 and WO95/02697) and the L5 region (WO95/02697). An adenovirus, according to the present invention, may contain a deletion or multiple deletions, for example, a deletion in the E1 and E4 regions. Further, an adenovirus of the invention may contain a deletion in the E1 region into which a nucleic acid of interest is inserted.

A nucleic acid sequence of particular interest is one which encodes an antigen to which an immune response is directed. Antigens which may be encoded by such sequences include, but are not limited to, antigenic epitopes or proteins from cancerous cells (*e.g.*, tumor cell surface-specific proteins), viruses, fungi, bacteria, protozoa, or mycoplasma. In one embodiment, antigens are derived from enveloped or non-enveloped viruses. In another embodiment, antigens are derived from viruses including, but not

limited to, those from the family Arenaviridae (*e.g.*, Lymphocytic choriomeningitis virus), Arterivirus (*e.g.*, Equine arteritis virus), Astroviridae (Human astrovirus 1), Birnaviridae (*e.g.*, Infectious pancreatic necrosis virus, Infectious bursal disease virus), Bunyaviridae (*e.g.*, California encephalitis virus Group), Caliciviridae (*e.g.*, Caliciviruses), Coronaviridae (*e.g.*, Human coronaviruses 299E and OC43), Deltavirus (*e.g.*, Hepatitis delta virus), Filoviridae (*e.g.*, Marburg virus, Ebola virus Zaire), Flaviviridae (*e.g.*, Yellow fever virus group, Hepatitis C virus), Hepadnaviridae (*e.g.*, Hepatitis B virus), Herpesviridae (*e.g.*, Epstein-Bar virus, Simplexvirus, Varicellovirus, Cytomegalovirus, Roseolovirus, Lymphocryptovirus, Rhadinovirus), Orthomyxoviridae (*e.g.*, Influenzavirus A, B, and C), Papovaviridae (*e.g.*, Papillomavirus), Paramyxoviridae (*e.g.*, Paramyxovirus such as human parainfluenza virus 1, Morbillivirus such as Measles virus, Rubulavirus such as Mumps virus, Pneumovirus such as Human respiratory syncytial virus), Picornaviridae (*e.g.*, Rhinovirus such as Human rhinovirus 1A, Hepatovirus such Human hepatitis A virus, Human poliovirus, Cardiovirus such as Encephalomyocarditis virus, Aphthovirus such as Foot-and-mouth disease virus O, Coxsackie virus), Poxviridae (*e.g.*, Orthopoxvirus such as Variola virus), Reoviridae (*e.g.*, Rotavirus such as Groups A-F rotaviruses), Retroviridae (Primate lentivirus group such as human immunodeficiency virus 1 and 2), Rhabdoviridae (*e.g.*, rabies virus), Togaviridae (*e.g.*, Rubivirus such as Rubella virus), Human T-cell leukemia virus, Murine leukemia virus, Vesicular stomatitis virus, Wart virus, Blue tongue virus, Sendai virus, Feline leukemia virus, Simian virus 40, Mouse mammary tumor virus, or Dengue virus.

In a further embodiment, an antigen is derived from *Streptococcus agalactiae, Legionella pneumophilia, Streptococcus pyogenes, Escherichia coli, Neisseria gonorrhosae, Neisseria meningitidis, Pneumococcus, Hemophilis influenzae* B, *Treponema pallidum,* Lyme disease spirochetes, *Pseudomonas aeruginosa, Mycobacterium leprae, Brucella abortus, Mycobacterium tuberculosis, Plasmodium falciparum, Plasmodium vivax, Toxoplasma gondii, Trypanosoma rangeli, Trypanosoma cruzi, Trypanosoma rhodesiensei, Trypanosoma brucei, Schistosoma mansoni, Schistosoma japanicum, Babesia bovis, Elmeria tenella, Onchocerca volvulus, Leishmania tropica, Trichinella spiralis, Theileria parva, Taenia hydatigena, Taenia ovis, Taenia saginata, Echinococcus granulosus, Mesocestoides corti, Mycoplasma arthritidis, M. hyorhinis, M. orale, M. arginini, Acholeplasma laidlawii, M. salivarium, M. pneumoniae, Candida albicans, Cryptococcus neoformans, Histoplasma capsulatum, Coccidioides immitis, Blastomyces dermatitidis, Aspergillus fumigatus,* or *Penicillium marneffei.*

Suitable nucleic acid sequences encoding antigens are well-known to those of skill in the art and may be identified from the GENBANK® or EMBL databases. The nucleic acid sequences may encode a protein, peptide, or epitope of an antigen and may have exogenous or endogenous expression control sequences, such as an origin of replication, a promoter, an enhancer, or necessary information processing sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Construction of such nucleic acid sequences is well-known in the art and is described further in Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

(1989). Preferred expression control sequences may be promoters derived from metallothionine genes, actin genes, immunoglobulin genes, CMV, SV40, adenovirus, bovine papilloma virus, and the like.

An adenovirus of the invention may be prepared as exemplified herein or by any technique known to one of skill in the art (see, for example, Levreto, et al. (1991) *Gene* 101:195; EP 185 573; Graham (1984) *EMBO J.* 3:2917). In particular, they may be prepared by homologous recombination between an adenovirus and a plasmid which carries, *inter alia*, the nucleic acid sequence of interest. The homologous recombination is effected following cotransfection of the said adenovirus and plasmid into an appropriate cell line. The cell line which is employed should preferably be transformable by the said elements, and contain the sequences which are able to complement the part of the genome of the replication-defective adenovirus, preferably in integrated form in order to avoid the risks of recombination. Examples of cell lines which may be used include the human embryonic kidney cell line 293 (Graham, et al. (1977) *J. Gen. Virol.* 36:59) which contains, in particular, integrated into its genome, the left-hand part of the genome of an Ad5 adenovirus (12%), or cell lines which are able to complement the E1 and E4 functions (see, *e.g.*, WO94/26914 and WO95/02697). Subsequently, the adenoviruses which have multiplied are recovered and purified using standard molecular biological techniques, as illustrated in the examples.

The present invention also includes pharmaceutical compositions containing one or more adenoviruses dispersed in a physiologically acceptable medium, which is preferably buffered to physiologically normal pH. Such pharmaceutical compositions, in accordance with the present invention are

formulated for administration by oral routes. Preferably the pharmaceutical composition or pharmaceutical preparation contains an efficacious dose of an adenovirus and a pharmaceutically acceptable carrier. Oral administration of the pharmaceutical composition may be in the form of pills, tablets, lacquered tablets, coated tablets, granules, hard and soft gelatin capsules, solutions, paste, gel, solid or semi-solid form, syrups, emulsions, suspensions or aerosol mixtures.

The selected pharmaceutically acceptable carrier may be an inert inorganic and/or organic carrier substance and/or additive. For the production of pills, tablets, coated tablets and hard gelatin capsules, the pharmaceutically acceptable carrier may include lactose, cornstarch or derivatives thereof, talc, stearic acid or its salts, and the like. Pharmaceutically acceptable carriers for soft gelatin capsules include, for example, fats, waxes, semisolid and liquid polyols, natural or hardened oils, and the like. Suitable carriers for the production of solutions, emulsions, or syrups include, but are not limited to, water, alcohols, glycerol, polyols, sucrose, glucose, and vegetable oils. Suitable carriers for microcapsules include copolymers of glycolic acid and lactic acid.

In addition to an adenovirus and a pharmaceutically acceptable carrier, the pharmaceutical composition may contain an additive or auxiliary substance. Exemplary additives include, for example, fillers, disintegrants, binders, lubricants, wetting agents, stabilizers, emulsifiers, preservatives, sweeteners, colorants, flavorings, aromatizers, thickeners, diluents, buffer substances, solvents, solubilizers, salts for altering the osmotic pressure, coating agents or antioxidants. A

generally recognized compendium of methods and ingredients of pharmaceutical compositions is Remington: The Science and Practice of Pharmacy, Alfonso R. Gennaro, editor, 20th ed. Lippingcott Williams & Wilkins: Philadelphia, PA, 2000.

Further, the adenovirus may be administered with an adjuvant to enhance a subject's T cell response to the antigen. Examples of such adjuvants include, but are not limited to, aluminum salts; Incomplete Freund's adjuvant; threonyl and n-butyl derivatives of muramyl dipeptide; lipophilic derivatives of muramyl tripeptide; monophosphoryl lipid A; 3'-de-O-acetylated monophosphoryl lipid A; cholera toxin; phosphorothionated oligodeoxynucleotides with CpG motifs and adjuvants disclosed in U.S. Patent No. 6,558,670.

Dosage and administration are adjusted to provide sufficient levels of the adenovirus or to maintain the desired effect of preventing or reducing signs or symptoms of a disease or infection, or reducing severity of a disease or infection. Factors which may be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Such factors may be assessed by a physician or qualified medical professional and the amount adjusted accordingly. Preferably, an effective amount of adenovirus is administered such that a measurable immune response is induced upon exposure to said adenovirus. A measure B cell response may be determined by, for example, production of antibodies to the antigen, and elicitation of a T cell response may be determined, for example, by measuring the production of cytokines, *e.g.*, IFN-gamma, IL-2, IL-4, IL-5, or IL-10.

Generally, the recombinant adenoviruses according to the invention are formulated and administered in the form of doses of between about 10^4 and about 10^{14} pfu or between about 10^5 to 10^{16} viral particles. Preferably, the doses of adenovirus are from about 10^5 to about 10^{10} pfu or about 10^6 to about 10^{12} viral particles. The term pfu (plaque-forming unit) corresponds to the infective power of a suspension of virions and is determined by infecting an appropriate cell culture and measuring, generally after 48 hours, the number of plaques of infected cells. The techniques for determining the pfu titer of a viral solution are well documented in the literature. In cases where administration of the first and second adenovirus are part of a vaccination protocol, the first adenovirus or priming advenovirus may be administered orally, parenterally injected (such as by intraperitoneal, subcutaneous, or intramuscular injection), or topically using well-known formulations and amounts to induce an immune response to said first adenovirus and antigens encoded thereby. Topical application may be carried out by intranasal administration (*e.g.*, by use of dropper, swab, or inhaler which deposits a pharmaceutical formulation intranasally) or direct contact with the skin such as in a cream, ointment, or gel. The first adenovirus may be given as a single dose schedule, or preferably in a multiple dose schedule. A multiple dose schedule is one in which a primary course of administration may be with 1-10 separate doses, followed by other doses (*i.e.*, second adenovirus or boost adenovirus) given at subsequent time intervals required to maintain and or reinforce the immune response, for example, at 1 to 4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage regimen will also, at least in part, be determined by the need of the

individual and be dependent upon the judgement of the practitioner.

The present invention offers a novel and very efficient means for inducing an immune response to an adenoviral vector in a subject and may be used as part of a vaccine or therapy for humans or other animal such as sheep, cattle, domestic animals (*e.g.*, dogs and cats), and fish.

Another aspect of the present invention is a method for inducing a mucosal immune response to an antigen. The method involves orally administering an effective amount of a first adenovirus containing nucleic acid sequences encoding an antigen, and subsequently orally administering an effective amount of a second adenovirus containing said nucleic acid sequences encoding said antigen. In this method of the invention, an effective amount of adenovirus containing an antigen is administered in an amount which results in a measurable mucosal immune response. A mucosal immune response involves the production of mucosa-related IgA and a complement of T cells with mucosa-specific regulatory or effector properties and provides for host defense at the mucosal surfaces. For a more complete review of the mucosal immune system see Strober and James, "The Mucosal Immune System" In Basic & Clinical Immunology 8th Edition eds Stites, Terr, Parslow, (Appleton & Lange, 1994), pgs 541-551.

Further contemplated is a method of preferentially inducing immunoglobulin isotypes to an antigen. When a first and second advenovirus are administered orally to a subject and are derived from an adenovirus which naturally infects said subject (*i.e.*, homologous), an immunoglobulin of the isotype IgA is preferentially induced. When a first and second advenovirus are administered orally to a subject

and are derived from an adenovirus which does not naturally infect said subject (*i.e.*, heterologous), an immunoglobulin of the isotype IgG2a is preferentially induced. Further, when a first and second adenovirus are administered orally to a subject and are derived from at least two different adenoviruses, a heterologous and homologous adenovirus, immunoglobulins of the isotypes IgA and IgG2a subclass are induced in approximately equal amounts. As indicated herein, an equal or balanced amount of isotypes may be induced independent of the sequence of administration of heterologous or homologous adenovirus.

Preferential induction of immunoglobulin isotypes may be useful in cases where it may be beneficial to induce one isotype over the other, for example, induction of IgG isotypes to prevent recrudescence (Ramakrishna, et al. (2003) J. Virol. 77(8):4670-8) or induction of IgA for common mucosal infections (Svanborg, et al. *In:* Handbook of Mucosal Immunology, Orga et al., eds.).

The invention is described in greater detail by the following non-limiting examples.

Example 1: Mice, Cell Lines and Viruses

Female inbred C57Bl/6 mice and outbred ICR mice were used at 6-12 weeks of age.

BHK-21 and 293 cells were maintained in Dulbeccos' modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics.

AdHu5rab.gp recombinant, E-1 deleted Ad recombinant of the human serotype 5 expressing the glycoprotein of the ERA strain of rabies virus is well-known in the art (*Belyakov, et al. (1999)* Proc. Natl. Acad. Sci. USA *96:4512-4517*). E1-deleted AdC68rab.gp vaccine expressing the same transgene product in a simian Ad virus vector is also well-known in

the art (Farina, et al. (2001) *supra;* Xiang, et al. (2002) *supra*). Viral recombinants, as well as wild-type AdHu5 virus, were propagated and titrated on 293 cells. The ERA strain of rabies virus was grown on BHK-21 cells, purified by gradient centrifugation and inactivated by treatment with beta-propiolactone (BPL) (Wiktor, et al. (1978) *Dev. Biol. Stand.* 40:171-178). The protein content of the inactivated virus (ERA-BPL) was determined and adjusted to 1 mg/ml. The rabies stain CVS-11 used for challenge was propagated and titrated on BHK-21 cells.

Example 2: Immunization and Challenge of Mice

Mice were immunized once or twice with various doses, indicated in plaque forming units (pfu), of the AdHu5 or AdC68 constructs given per os or intramuscularly (i.m.). Mice immunized with an Ad viral recombinant expressing a viral antigen not derived from rabies virus are unable to induce rabies virus-specific antibodies (Xiang, et al. (2002) *supra*). Thus, this control was not included in the experiments conducted herein. Oral immunization with 10^6 pfu of the AdHu5rab.gp vaccine fails to induce serum antibody titers to rabies virus. Thus, the vaccination procedure was modified by applying the vaccines with a feeding tube to ensure swallowing rather than inhalation or spillage of the vaccine. Furthermore, the vaccine was diluted in a buffered salt solution rather than in saline. Mice were immunized with wild-type AdHu5 virus given intranasally or i.m. Mice were challenged with 10 mean lethal doses (LD_{50}) of the CVS-11 strain of rabies virus injected directly into the brain. Experiments were conducted 2-5 times in groups of 5-8 mice to ensure reproducibility.

Example 3: Preparation of Samples

Blood was harvested by retro-orbital puncture. Sera were prepared and heat-inactivated at 56°C for 30 minutes. Sera were tested for rabies virus-neutralization starting at a 1:5 dilution and for neutralization of AdHu5 virus starting at a 1:20 dilution. Analysis was conducted by ELISA starting with a 1:200 dilution. Antibody isotypes were tested with a 1:800 dilution of sera. Vaginal lavage was harvested by rinsing the vaginal cavity three times with 50 µl of saline for a final volume of 150 µl. The sample was centrifuged at 5000 rpm for 5 minutes to remove debris. Vaginal lavage was titrated starting at a dilution of 1:2; antibody isotypes were determined with a 1:8 dilution (Xiang, et al. (1999) *J. Immunol.* 162:6716-6723). Feces was collected and suspended at 50 mg/ml in phosphate buffered saline (PBS) containing 1% NaN_3. After a one hour incubation at room temperature, samples were vortexed and debris was removed by centrifugation (Xiang and Ertl (1999) *supra*). Samples were tested for antibody titers starting at a 1:2 dilution and for isotypes at a 1:5 dilution.

Spleens, cervical and mesenteric lymph nodes and Peyers' patches were harvested 18 - 72 hours after oral immunization.

Example 4: Enzyme-Linked Immunoadsorbent Assay

Sera, vaginal lavage and fecal suspensions were tested on rabies virus-coated plates using well-established methods (Xiang, et al. (1999) *supra*). Briefly, round-bottom microtiter plate wells were coated overnight with 0.2 µg of ERA-BPL virus or purified AdHu5 virus diluted in 100 µl of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3mM Na_2N, pH 9.6). Plates were subsequently treated for 24 hours with PBS containing 3% bovine serum albumin (BSA). The following

day plates were washed two times with 150 µl of PBS for 24 hours, dried and stored at -20°C. Sera were serially diluted in PBS containing 3% BSA. The different dilutions of sera were incubated in duplicate at 100 µl per well on the ERA-BPL coated plates for 1 hour at 4°C. Fecal suspensions and vaginal lavage were incubated overnight. Plates were washed five times with PBS and treated with an alkaline phosphatase-conjugated, goat anti-mouse antibody for 1 hour at 4°C. Plates were washed and incubated for 20 minutes with the substrate (10 mg d-nitrophenyl phosphate disodium dissolved in 10 ml of 1 mM $MgCl_2$, 3mM NaN_3, 0.9 M diethanolamine, pH 9.8). Plates were then measured in an automated ELISA reader at 405 nm. Isotypes of antibodies to rabies virus were tested on ERA-BPL-coated plates with the Calbiochem isotyping kit, which has comparable sensitivity for different antibody isotypes (Vos, et al. (2001) *supra*).

Example 5: Virus Neutralization Assay

Sera were tested for neutralization of CVS-11 virus, which is closely related, antigenically, to the ERA virus (Wiktor (1973) *WHO Monogr. Ser.* 23:101-123). Sera were tested for neutralization of AdHu5 virus by a plaque reduction assay (Farina, et al. (2001) *supra*).

Example 6: Reverse Transcription Polymerase Chain Reaction

Mice were sacrificed and lymphoid tissues were harvested and disrupted by a polytron probe in a solution of Tri-reagent (MRC, Cincinnati, OH). RNA was isolated from individual samples as recommended by the manufacturer. Briefly, 100 µl of BCP solution (MRC, Cincinnati, OH) was added to each sample. The aqueous phase was transferred to fresh tubes, and RNA was precipitated by isopropanol, washed with 70% ethanol and resuspended in DEPC-treated

water (Ambion, Inc., Houston, TX). DNA was removed by treatment with DNAse (Ambion, Inc. Houston, TX) for 30 minutes at 37°C. DNAse was removed with the DNAse removal kit (Ambion, Inc., Houston, TX). Complementary DNA (cDNA) was synthesized from RNA samples with M-MLV Reverse Transcriptase (Life Technologies, Inc., Rockville, MA). Samples were amplified for rabies virus glycoprotein and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNAs using the following primers: rab.gp - forward, 5'-AAA GCA TTT CCG CCC AAC AC-3' (SEQ ID NO:1); rab.gp - reverse, 5'-GGT TAC TGG AGC AGT AGG TAG A-3' (SEQ ID NO:2); GAPD - forward, 5'-GGT GAA GGT CGG TGT GAA CGG ATT T-3' (SEQ ID NO:3); and GAPDH - reverse 5'-AAT GCC AAA GTT GTC ATG GAT GAC C-3' (SEQ ID NO:4). PCR conditions for all genes included an initial denaturation at 94°C for 5 minutes and 40 cycles of: denaturation at 94°C for 1 minutes, annealing at 55°C for 2 minutes and extension at 72°C for 3 minutes. The amplicons were separated by electrophoresis on a 1% agarose gel, visualized by ethidium bromide, and analyzed on a Fluorimager SI (Vistra Fluorescence).

What is claimed is:

1. A method for inducing an immune response in a subject pre-exposed to an adenovirus comprising orally administering to a subject, that has been exposed to a first adenovirus, an effective amount of a second adenovirus so that an immune response to the second adenovirus is induced.

2. The method of claim 1, wherein the first adenovirus and said second adenovirus contain a nucleic acid sequence encoding an antigen to which the immune response is directed.

3. The method of claim 1, wherein the first adenovirus and the second adenovirus comprise a vaccine.

4. A method for inducing a mucosal immune response to an antigen comprising:

orally administering an effective amount of a first adenovirus containing nucleic acid sequences encoding an antigen, and

orally administering an effective amount of a second adenovirus containing said nucleic acid sequences encoding said antigen,

so that a mucosal immune response is induced.

5. The method of claim 4, wherein the first adenovirus and second adenovirus comprise a vaccine.

ABSTRACT

The present invention provides a method for inducing an immune response to an adenovirus in a subject which has been pre-exposed to an adenovirus. The invention further provides a method for inducing a mucosal immune response to an antigen. The methods of the invention are carried out by oral administration of adenovirus.

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